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Video Article

# Rapid Analysis of Circadian Phenotypes in Arabidopsis Protoplasts Transfected with a Luminescent Clock Reporter

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## Abstract

The plant circadian clock allows the anticipation of daily changes to the environment. This anticipation aids the responses to temporally predictable biotic and abiotic stress. Conversely, disruption of circadian timekeeping severely compromises plant health and reduces agricultural crop yields. It is therefore imperative that we understand the intricate regulation of circadian rhythms in plants, including the factors that affect motion of the transcriptional clockwork itself.

Testing circadian defects in the model plant *Arabidopsis thaliana* (*Arabidopsis*) traditionally involves crossing specific mutant lines to a line rhythmically expressing firefly luciferase from a circadian clock gene promoter. This approach is laborious, time-consuming, and could be fruitless if a mutant has no circadian phenotype. The methodology presented here allows a rapid initial assessment of circadian phenotypes. Protoplasts derived from mutant and wild-type *Arabidopsis* are isolated, transfected with a rhythmically expressed luminescent reporter, and imaged under constant light conditions for 5 days. Luminescent traces will directly reveal whether the free-running period of mutant plants is different from wild-type plants. The advantage of the method is that any *Arabidopsis* line can efficiently be screened, without the need for generating a stably transgenic luminescent clock marker line in that mutant background.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/54586/>

## Introduction

Most living organisms possess an endogenous timekeeper to aid efficient negotiation of daily changes to the environment. This timekeeper, the circadian clock, regulates many aspects of the metabolism and physiology of an organism to anticipate predictable changes in the external environment. In plants for example, anticipation of temporally predictable pathogen or herbivore attacks strongly reduces overall susceptibility<sup>1-4</sup>. Starch metabolism is tightly regulated by the circadian clock to ensure that starch reserves last until dawn whether grown at long or short day conditions<sup>5</sup>. Indeed, plants with circadian clocks matching the 24 hr environment show increased growth rates, carbon fixation rates and survival rates compared to plants running a clock of mismatched period<sup>6</sup>. The extensive influence of circadian rhythms in all these processes arises to a large extent from the rhythmic regulation of up to a third of the total transcriptome in *Arabidopsis*<sup>7</sup>. These rhythmic gene products are involved in a broad range of cellular processes including metabolic pathways, hormone signaling, and stress responses<sup>7</sup>. On top of that, direct circadian regulation of protein function is established by circadian regulation of phosphorylation<sup>8</sup> and most probably other post-translational modifications (PTMs).

At the center of the circadian clock that drives this daily transcriptional reprogramming lies a network of transcriptional/translational feedback loops (TTFLs), including the morning-expressed genes *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) that repress the expression of the evening genes *TIMING OF CAB 1* (*TOC1*), *GIGANTEA* (*GI*) and the members of the evening complex (EC); *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3* (*ELF3*), and *ELF4*<sup>9-11</sup>. Together with the *PSEUDO RESPONSE REGULATOR*-genes (*PRR3*, *5*, *7*, and *9*), *TOC1* represses *CCA1/LHY* expression whereas the EC acts as a positive regulator<sup>12-14</sup>. Additional to feedback mechanisms, post-transcriptional and post-translational regulation of the TTFL network components plays an important role in tuning circadian rhythms. To date, the most abundant PTM identified on circadian clock proteins is phosphorylation<sup>15</sup>. The phosphorylation of *CCA1* by Casein Kinase 2 (CK2) affects its binding to target promoters<sup>16</sup> and is important for temperature compensation of the circadian clock<sup>17</sup>. The *PRR* proteins are phosphorylated differentially over the circadian cycle, and phosphorylation of *TOC1* affects interaction with its negative regulator, the evening-phased clock component *ZEITLUPE* (*ZTL*)<sup>18</sup>. These examples illustrate how PTMs and protein-protein interactions tune the TTFL network into a 24-hr transcriptional oscillator. For a more detailed introduction to the transcriptional clock network and its regulation, excellent recent reviews are available (e.g. reference<sup>15</sup>).

However, what remains less clear is the extent to which rhythmically regulated processes and other aspects of cellular metabolism feed back into the timekeeping mechanism itself. Extensive screening of *Arabidopsis* mutants for clock defects could gain further understanding of which

mechanisms or signaling pathways could be involved in the generation of 24 hr rhythms from a TTFL network. To this end, Kim and Somers previously published a break-through method for the efficient and rapid analysis of clock function in any Arabidopsis line<sup>19</sup>. Based on the use of transient expression in protoplasts, this methodology surpasses the need for stable transgenic lines or crosses to luminescent clock marker lines. Here, we visualize a high yielding protoplast isolation method<sup>20</sup> combined with an optimized protocol to screen circadian defects by luminescent imaging of transiently expressed clock markers in a 96-well plate reader.

We will compare wild-type Col-0 Arabidopsis to well-characterized clock mutants to confirm the methodology described here successfully detects altered circadian rhythms. Protoplasts derived from these lines are transfected with a reporter construct that expresses firefly luciferase from the circadian CCA1 promoter; *CCA1pro:LUC*<sup>19</sup>. Luciferase catalyzes the multistep reaction in which luciferin is converted to electronically excited oxyluciferin that emits light<sup>21</sup>, which is imaged over time to assess the period, amplitude and phase of transcriptional rhythms in these protoplasts.

The protocol consists of three major parts; isolating the protoplasts, transfecting the protoplasts with the reporter plasmid, and luminescent imaging. These three parts should always be performed on a single day, using freshly prepared buffers and reagents. Plant growth and purification of sufficient amounts of reporter plasmid should be performed in advance and are not visualized in this protocol.

## Protocol

NOTE: In this protocol, reagents and volumes described are expressed per Arabidopsis line that is tested, and will result in six replicate wells for that genotype. Multiply the materials with the number of lines to be analyzed when analyzing more than one line. In the video, wild-type Arabidopsis will be compared to the circadian clock mutant line *ztl* (although representative results from additional lines will be provided subsequently).

Enzyme solution	
Cellulase	0.5% (w/v)
Pectinase R10	0.25% (w/v)
D-mannitol	400 mM
CaCl <sub>2</sub>	10 mM
KCl	20 mM
Bovine Serum Albumin	0.1% (w/v)
MES, pH 5.7	20 mM
W5 solution	
NaCl	150 mM
CaCl <sub>2</sub>	125 mM
KCl	5 mM
MES, pH 5.7	2 mM
Glucose	5 mM
MMg solution	
MES, pH 5.7	4 mM
D-mannitol	400 mM
MgCl <sub>2</sub>	15 mM
PEG solution	
PEG4000	40% (w/v)
D-mannitol	200 mM
CaCl <sub>2</sub>	100 mM
Imaging solution	
W5 solution	to final volume
Fetal Bovine Serum	5% (v/v)
Luciferin	1.2 mM
Ampicillin	50 mg/ml

**Table 1: List of solutions.**

## 1. Preparation of Materials and Buffers

### 1. Plant Materials

1. Keep *Arabidopsis Col-0* seeds in water in a 1.5 ml tube at 4 °C in the dark for four days. Pipette the seeds onto soil in a pot and transfer to long day conditions (16 hr light 8 hr dark) at 21 °C for 7 days. Leave a lid on the pot for the first three days to ensure high humidity. Transplant six seedlings to a new 8 cm x 13 cm x 5 cm pot and continue growing the plants under the same conditions for another 21 days. Make sure the soil is moist throughout.

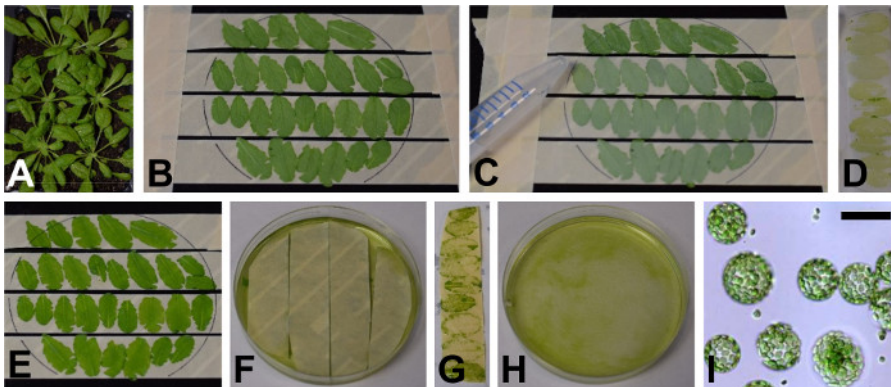
### 2. Reporter Plasmid

1. Purify the *CCA1pro::LUC* reporter plasmid<sup>19</sup> from bacterial culture in advance, using a DNA isolation kit according to manufacturer's instructions.  
NOTE: 20 µg reporter plasmid is required (10 µl at 2 µg/µl in dH<sub>2</sub>O) for a transfection.

### 3. Solutions

NOTE: For this protocol five solutions are needed: Enzyme solution, Wash 5 (W5) solution, Mannitol-Magnesium (MMg) solution, polyethylene glycol (PEG) solution, and imaging solution (**Table 1**). Prepare these before continuing to the protoplast isolation step.

1. Prepare 10 ml of enzyme solution as per **Table 1**, adding the enzymes last, and filter sterilize the solution through a 0.22 µm syringe filter.
2. Prepare the remaining solutions in the following volumes: 15 ml W5 solution, 10 ml MMg solution, 1 ml PEG solution and 1.5 ml imaging solution as per **Table 1**.  
NOTE: It is important that the PEG solution is prepared no less than an hr before the transfection to ensure that the PEG has fully dissolved. Filter sterilize the imaging solution through a 0.22 µm syringe filter.



**Figure 1: Protoplast isolation.** The leaves of adult *Arabidopsis* plants (**A**) are fixed on autoclave tape with the lower epidermal layer facing up (**B**). (**C**) Magic tape is gently pressed onto the lower epidermal layer with the tip of a 15 ml conical tube. (**D**) The magic tape is removed to expose the mesophyll cells (**E**). (**F**) Strips of autoclave tape with leaves attached are floated on enzyme solution containing cellulase and pectinase, releasing protoplasts into the solution. (**G**) The leaf skeletons remain on autoclave tape after enzyme digestion and are discarded, leaving the protoplasts in enzyme solution (**H**). (**I**) Final resulting mesophyll protoplasts (Scale bar = 50 µm). [Please click here to view a larger version of this figure.](#)

## 2. Protoplast Isolation

1. Label a petri dish and add 10 ml filter-sterilized Enzyme solution.
2. Fix four strips of autoclave tape with the sticky side up on a clean lab surface, and mark the size of the petri dish on the strips.
3. Cut leaves of 4-week old plants (**Figure 1a**), and press the upper epidermis onto the autoclave tape (*i.e.* the lower epidermal surface facing up (**Figure 1b**)).
4. Gently press strips of magic tape onto the lower epidermal surface using a 15 ml conical tube. Take care not to crush the leaf tissue (**Figure 1c**). Carefully pull the magic tape off to strip the lower epidermis (**Figure 1d**) and expose the mesophyll cells (**Figure 1e**).
5. Cut the autoclave tape to fit the petri dish, use tweezers to move the tape into the petri dish and float on the enzyme solution (**Figure 1f**), leaves facing down. Rotate at 60 rpm on a platform shaker for 60 min, during which the protoplasts will be released into the solution.
6. Use tweezers to remove and discard the strips of autoclave tape (**Figure 1g**) and pipette the solution containing the protoplasts (**Figure 1h**) into a 50 ml conical tube. Use a wide-bore pipette tip (such as a P5000 tip or a P1000 tip with the end cut off).
7. Centrifuge the protoplasts at 100 x g for 3 min at 4 °C and remove the supernatant with a pipette. Take care, as the pellet is very loose.
8. Add 10 ml W5 solution to the protoplasts and resuspend by gently swirling the tube.
9. Rest the protoplasts on ice for 30-60 min. During this step, assess yield by counting protoplasts in a hemocytometer (**Figure 1i**).
10. Collect the protoplasts by centrifugation at 100 x g for 3 min at 4 °C and remove the supernatant with a pipette. Take care, as the pellet is very loose.
11. Resuspend the protoplasts to a concentration of  $5 \times 10^5$  protoplasts/ml in MMg solution.

### 3. Protoplast Transfection

1. Add 10  $\mu$ l reporter plasmid (2  $\mu$ g/ $\mu$ l) to a 15 ml conical tube.
2. Add 400  $\mu$ l protoplasts to the tube.
3. Add one volume (410  $\mu$ l) PEG solution and mix by inverting the tube gently 12 times to transfect the protoplasts.
4. After 8-15 min incubation at room temperature, dilute the protoplast-DNA-PEG mixture with four volumes (1640  $\mu$ l) W5 solution and mix by inverting gently six times.
5. Collect the transfected protoplasts by centrifugation at 100 x g for 2 min at room temperature and remove the supernatant with a pipette. Once again, take care, as the pellet is very loose.
6. Resuspend the protoplasts in 1,250  $\mu$ l imaging solution.
7. Aliquot 200  $\mu$ l into six replicate wells of a 96-well plate, fill any empty wells with W5 solution, and seal the plate with an adhesive clear lid suitable for luminescent imaging.

### 4. Imaging

1. Transfer the plate to any luminescent imaging setup suitable for plant imaging. Change the adhesive lids on the plates 10-15 min after transfer to avoid condensation and pressure differences among the wells, then start imaging. Read the plate every ~45 min (3 sec per well) for five days at room temperature.  
NOTE: The frequency of plate reads and the read time per well might vary depending on the imaging setup.

### 5. Data Analysis

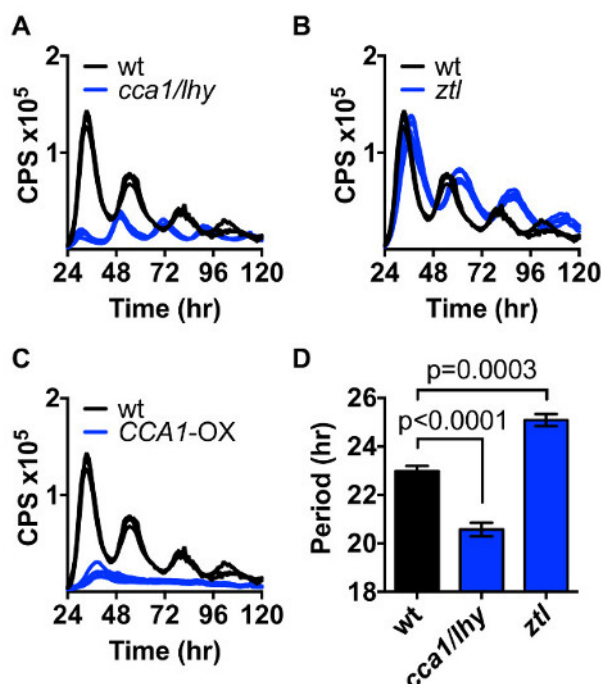
1. Analyze the luminescence results using any analysis software (for example, Biological Rhythms Analysis Software System (BRASS)<sup>22</sup>). Compare the mutant line to wild-type controls to reveal circadian defects.

## Representative Results

Protoplasts of wild-type *Arabidopsis* were compared to the known clock mutants *cca1/lhy* (short period mutant<sup>2</sup>), *ztl* (long period mutant<sup>23</sup>), and the overexpression line *CCA1-OX* (arrhythmic<sup>24</sup>). All were transiently transfected with the *CCA1pro:LUC* reporter and imaged in constant light on a plate reader over 5 days.

Here we show that in the *cca1/lhy* mutant, the free-running period of circadian-controlled gene expression is shortened (**Figure 2a**), in line with the published period analyzed by stable transgenic expression of luminescent clock markers<sup>2,9</sup>. The period of circadian oscillations in protoplasts of the *ztl* mutant is longer than in wild type (**Figure 2b**), in accordance with previously published results from seedlings, cell suspension cultures and protoplasts<sup>19,23,25</sup>.

Overexpression of CCA1 locks the clock in morning-phase and global transcription becomes arrhythmic<sup>24</sup>. Consistently, we observed no oscillations in *CCA1pro:LUC* expression in *CCA1-OXprotoplasts* (**Figure 2c**).



**Figure 2: Protoplast Circadian Rhythms** Protoplasts of Col-0, *cca1/lhy*, *ztl*, and *CCA1-OX* were transiently transformed with the *CCA1pro:LUC* construct and circadian rhythms in luminescence were imaged over 5 days (A-C). (D) Circadian period estimates based on luminescence traces in Col-0, *cca1/lhy* and *ztl* (A-B). Mean  $\pm$  SEM, n = 3-5, t-test p-value as indicated. [Please click here to view a larger version of this figure.](#)

Genotype	Period (protoplasts)	Period (transgenic)	Reported by:	References
Col-0	23.0 $\pm$ 0.21	24.4 $\pm$ 0.09	<i>pCCA1:LUC</i> in Col-0	(2)
<i>cca1/lhy</i>	20.6 $\pm$ 0.13	19.9 $\pm$ 0.11	<i>pCCA1:LUC</i> in Col-0	(2)
		~18	RNA in Ler background	(9)
<i>ztl</i>	25.1 $\pm$ 0.12	27.4 $\pm$ 0.5	<i>CAB2:LUC</i> in C24 ecotype	(22)
<i>CCA1-OX</i>	arrhythmic	arrhythmic	<i>pCCA1:LUC</i> (LD data only)	(2)
		arrhythmic	RNA and protein in Col-0 background	(23)

**Table 2: Circadian Rhythms in Protoplasts Compared to Transgenic Seedlings.** The circadian period of *pCCA1:LUC* expression in protoplasts compared with the period lengths first reported for the mutation and, if available, with *pCCA1:LUC* expression in Col-0.

## Discussion

Here, we present a rapid assay to screen circadian period defects in Arabidopsis lines, including protoplast isolation, transfection, and luminescent imaging. The circadian period in wild-type Arabidopsis and the clock mutants *cca1/lhy*, *ztl*, and *CCA1-OX* was calculated from measurements of luminescence from a *CCA1pro:LUC* reporter and found to be consistent with published data generated from whole plants using more time-consuming transgenic approaches (Table 2). Using this assay to screen Arabidopsis mutants avoids the initial requirement to generate transgenic luminescent lines, which is time consuming and might only reveal that a particular mutant exhibits wild-type rhythms. A clear advantage of this protocol is that any Arabidopsis line can be screened in short time for altered circadian transcriptional rhythms, which will help the identification of more genes affecting timekeeping in plants.

Isolation of protoplasts can be laborious, especially if the mutant line displays a dwarfed phenotype. Previously reported methods for generating protoplasts involve cutting leaves or seedlings into thin strips and vacuum infiltrating the strips with enzyme solution to allow the enzymes to reach the cell walls<sup>26,27</sup>. The subsequent digestion requires at least 3 hr incubation in the dark to release the protoplasts into the enzyme solution. When vacuum infiltration is not applied, incubation time up to 18 hr is advised. In the protocol presented here, vacuum infiltration is unnecessary because the epidermal cell layer impenetrable to the enzymes is removed by tape. When generating protoplasts by cutting plant material it is necessary to separate protoplasts from cell wall debris after digestion; this is typically done by filtering the solution or purifying it on a sugar gradient<sup>27,28</sup>. Here, any undigested tissue will remain on the autoclave tape after digestion, so filtration and sugar gradient purification of the protoplasts can be omitted. There is a chance that the stress resulting from prolonged protoplasting procedures affects cell viability and the circadian clock. The tape-based protoplasting method<sup>20</sup> visualized here yields a high number of protoplasts; and given the viability of cells over a circadian time series and the matching period lengths of protoplasts and whole seedlings (Table 2), the methodology described here is preferred over alternative methods to generate protoplasts.



The transfection step of the protocol is a critical step that impacts on the viability of the protoplasts. The PEG solution enables the DNA to be delivered into the cell, and both incubation time in this solution (step 3.4) and the percentage of PEG should be determined empirically. The standard concentration is 20%, with lower concentrations reducing the transformation efficiency and higher concentrations reducing viability<sup>28</sup>. Incubation time as short as five minutes could yield efficient transfection of the protoplasts<sup>27</sup>.

The protoplasts can be imaged on any luminescent imaging platform. In this video, we have used a luminescence plate reader equipped with external lights (red and blue LEDs, 630 and 470 nm, respectively, at a combined intensity of ~20  $\mu$ E), which allows for high-throughput screening and frequent measurements. Other imaging equipment that detects luminescence, such as alternative plate readers or setups based around a charge-coupled device (CCD) camera, could be applied equally well as long as illumination is available for photosynthesis. The advantage of using a CCD camera mounted on a controllable cabinet is that light and temperature can be programmed. A disadvantage is the longer capture time, introducing prolonged periods of darkness that can cause stress to the protoplasts in addition to perturbing endogenous timekeeping. Regardless of which experimental platform is chosen, adjustment of settings is likely to be necessary compared to settings for seedlings or mature plants. In our experience, increasing the concentrations of reporter plasmid during transfection and/or luciferin in the imaging buffer might resolve any erratic or quickly dampening rhythms that might occur in initial experiments.

In future experiments, the methodology described here can be applied to study the circadian phenotype of any mutant Arabidopsis line. With minor modifications, the effect of e.g. various drugs on timekeeping could be studied in this setup. Furthermore, the transfection can be modified to include artificial microRNA for gene silencing<sup>19</sup>, further expanding the versatility of this protocol. Protocols to generate rice protoplasts are well-established<sup>29</sup> and the imaging protocol presented here could equally be applied to further our understanding of the clock in economically important monocot crop plants.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

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